

D R A

The Rapid Quantitation of the Filamentous Blue-Green  
Alga Plectonema boryanum by the Luciferase Assay for ATP

by

Valerie N. Bush  
Department of Biology  
Delaware State College  
Dover, Delaware 19901

March 1974

This research was funded by a NASA Research Grant to V. Bush  
at Delaware State College 08002-003 for 1973.

(NASA-CR-140486) THE RAPID QUANTITATION  
OF THE FILAMENTOUS BLUE-GREEN ALGA  
PLECTONEMA BORYANUM BY THE LUCIFERASE  
ASSAY FOR ATP (Delaware State Coll.)  
20 p HC \$4.50

N74-33527

CSCL 66C

G3/04

Unclas  
17031

## INTRODUCTION

Plectonema boryanum is a filamentous blue-green alga. Blue-green algae have a procaryotic cellular organization similar to bacteria, but are usually obligate photoautotrophs, obtaining their carbon and energy from photosynthetic mechanisms similar to higher plants. This research deals with a comparison of three methods of quantitating filamentous populations: microscopic cell counts, the luciferase assay for ATP and optical density measurements.

Microscopic cell counts are the most direct way of determining the number of cells present in a given population. Counting cells is very tedious so this is usually not the preferred method used in any research of this nature. Cell counts, though, are necessary in standardizing other quantitation techniques. In a filamentous cell culture, individual cells remain attached to each other after cell division. Surrounding each cell is a gelatinous sheath which encloses the cell wall of each cell and is the septum which connects adjoining abutted cells. The disruption of this sheath but not disruption of the cells is necessary so that individual cells or cell chains of no more than 2 - 3 cells per chain are obtained. This is a prerequisite for accurate cell counting.

Physical methods using ultrasonic equipment (Nusbaum, 1970) and the French press (Fay and Lang, 1971) have been used to disrupt the filaments. The equipment used in these procedures was not available to us, so other means to disrupt the filaments were attempted. These included heating, cooling, high frequency sound recording and replaying, and chemical treatments.

Adenosine triphosphate (ATP) is found in all living organisms. ATP measurements have been used to determine biomass in algal populations (Holm-Hansen, 1970). After extraction of ATP from cells, the amount of ATP present can be measured by the bioluminescent firefly luciferase reaction (McElroy, 1960). There is a direct linear relationship between ATP concentration and maximum intensity of light emitted in this reaction. This relationship exists over a range determined by a ratio of the concentration of luciferin-luciferase to that of ATP and by the sensitivity of the light measuring instruments.

Growth curves have been obtained for a variety of blue-green algae using Klett measurements, a turbidimetric measurement (Kratz and Myers, 1955, Nusbaum, 1970). In this study Spectronic-20 colorimetric readings were used as a measure of optical density.

## MATERIALS AND METHODS

Plectonema boryanum growth conditions. Cultures of P. boryanum were grown aseptically in modified Chu #10 broth (0.058 g  $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 0.29 g  $\text{NaNO}_3$ , 0.01 g  $\text{K}_2\text{HPO}_4$ , 0.025 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g  $\text{NaCO}_3$ , 0.044 g  $\text{NaSiO}_3 \cdot 5\text{H}_2\text{O}$ , 0.0035 g ferric citrate, 1000 ml distilled water) at  $30^\circ\text{C}$  in a Sheldon Climatarium (E.H. Sheldon Equipment Company, Muskegon, Michigan) under cool-white fluorescent lights. The Climatarium contained two 400 watt cool fluorescent bulbs each emitting 17,600 lumens at a distance of 2 feet from the flask. Five ml inoculations of 5 - 7 day old algal cells were made into 50 ml Chu broth and grown in 250 ml screw cap Erlenmeyer flasks. Five day old cultures were quantitated. The algal cultures were periodically checked for bacterial contamination; only bacteria free cultures were used.

Disruption of algal filaments. The algal cells were treated with ultrasonics ranging from 14,000 - 22,000 cycles per second. One ml of algal cells was placed in a cuvette. The cuvette was suspended in a twetter emitting the desired frequency for 5 seconds.

One ml of algal cells was placed in a test tube and heated to  $90^\circ\text{C}$ . Samples of the treated cells were examined at periodic intervals under the microscope to detect disassociation of the algal septum.

One ml of algae was placed in a clean disposable test tube. The sample was then frozen for 12 hours at  $-50^\circ\text{C}$ . After defrosting cell counts were made.

Fresh pineapple chunks were crushed in a mortar and pestle

and the juice removed after filtration. One tenth ml of this juice was added to 1 ml algal cells and allowed to stand for 1 hour. For a control 0.1 ml of distilled water was added to 1 ml algal cells.

Four ml of algal culture was mixed with 1 ml of nitric acid (0.05N, 0.1N, 0.2N, 0.5N, 1.0N) and allowed to sit for five minutes.

A 4 ml sample of algal culture was mixed with 1 ml NaOH solution (0.05N, 0.1N, 0.2N, 0.5N, 1.0N) and allowed to remain for 5 minutes.

One ml of algal culture was mixed with 1 ml Triton X-100 (0.1%, 0.25%, 0.5%, 1%, 2%, 10%) and allowed to remain for 5 minutes.

Quantitation I: Microscopic Cell Counts. Microscopic cell counts were made using a Levy-Hauser hemocytometer. The four corner squares and the central square were located; the cells present within these were counted. If no dilution of the algal population was made, the titer of the cells was calculated by multiplying the number of cells counted times  $5 \times 10^4$ . Cells were counted using the oil immersion lens of a Wolfe-Wetzlen microscope (Carolina Biological Supply Co).

Quantitation II: Luciferase assay for ATP. Five-tenths ml of algal cells was aseptically pipetted to a clean, disposable test tube and mixed with 0.1 ml of apyrase (40 mg apyrase (Sigma Co.) per ml 0.03M  $\text{CaCl}_2$ ) to hydrolyze soluble ATP. The sample was allowed to sit for 15 minutes before 0.1 ml  $\text{HNO}_3$  (1.5N, 2.0N or 2.5N) was added. Five minutes later, 4.3 ml de-ionized distilled water (Continental Co, Bladensburg, Md.) was added to bring the total volume to 5 ml.

Algal cells were centrifuged in a table top centrifuge (International Equipment Co., Boston, Mass.) at top speed for 5

minutes. The supernatant was removed and filtered through a 0.22  $\mu$ m Millipore filter using the Swinex filtering apparatus (Millipore Co.). The filtrate was treated in the same manner as the algal cell sample as described above; this was the blank for the experiment. One-half ml of the filtrate was also added to apyrase and nitric acid as described above except that 4.2 ml of deionized distilled water and 0.1 ml of ATP (1  $\mu$ g ATP/ml deionized distilled water) were added to the treated filtrate bringing the volume to 5 ml also.

The amount of ATP in the treated samples was determined by injection by needle and syringe of 0.1 ml of each sample into a cuvette containing 0.1 ml DuPont luciferin-luciferase mixture (1 vial powdered luciferin-luciferase + 1.5 ml of diluent (0.2M Tris, pH 8, 0.06019 g  $\text{MgSO}_4$ ). The amount of light emitted was measured by a Chem Glow Photometer (American Instrument Co., Silver Spring, Md.). A permanent record of this response was recorded on an X-Y recorder (Hewlett Packard). The response of the blank was subtracted from both the algal cell sample and ATP standard. Since the response for a known concentration of ATP is determined for each luciferase mixture used, the amount of intracellular ATP in the algal cells can be calculated.

Quantitation III: Optical density measurements. Dilutions of P. boryanum were made in sterile Chu broth. The optical density of samples of 10%, 25%, 50%, 75 % and 100% (undiluted) algal cells was determined in a Bausch and Lowe Spectronic 20 using the absorption wavelength of 600 nm.

Cell Volume. The volume of the algal cells was calculated by measuring the length and width of individual cells. The cylindrical

volume formula ( $V = \pi r^2 h$ ) and the formula for the volume of a rectangle ( $V = l w h$ ) were both used and the values averaged because it was assumed that the algal cells were actually a hybrid of these geometrical forms.

## RESULTS

Various methods were used to disrupt the algal filaments prior to microscopic counting. The setup devised to produce ultrasonic frequencies did not result in any change in the number of cells per filament in the algal population. The cells treated by heat were ruptured before septum rupture occurred, so no counts were made after this treatment. After the freezing treatment the cell chain length was not changed. The microscopic counts from the untreated sample were  $1.02 \times 10^8$  cells / ml and the frozen sample contained  $6.32 \times 10^7$  cells / ml.

The pineapple juice treatment had some effect on the length of the filamentous chains. The number of cells in each filament was not determined. The untreated samples counted had  $5.53 \times 10^7$  and  $5.17 \times 10^7$  cells per ml and the pineapple treated counts were  $3.32 \times 10^7$  and  $5.26 \times 10^7$  cells per ml respectively.

Nitric acid failed to disrupt the septum; destruction of the cell wall occurred prior to septum rupture. As the concentration of nitric acid increased, microscopic count values decreased (Table I). The destruction of algal cells was also seen by a comparison of Spectronic 20 readings taken at the same time microscopic counts were made.

Cell counts made after sodium hydroxide treatments and Triton X-100 treatments are presented in Tables II and III. Neither of these treatments disrupted the septum and had little effect on the cell walls.

Two normal nitric acid was found to be the optimal concentration



Table I: Effect of treating five day old *P. boryanum* cultures for five minutes with various concentrations of nitric acid

	Nitric Acid Concentration						
	0	0.01N	0.05N	0.1N	0.25N	0.5N	1.0N
Average microscopic cell counts ( x 10 <sup>6</sup> cells per ml)	29.4	26.5	27.0	13.9	13.3	9.77	9.55
Average Spectronic 20 values	.47	.41	.41	.21	.20	.16	.18

Table II: Effect of treating five day old P. boryanum cultures for five minutes with various concentrations of sodium hydroxide

	Sodium hydroxide concentration					
	0	0.05N	0.1N	0.2N	0.5N	1.0N
Microscopic Cell counts (x10 <sup>7</sup> cells per ml)	2.13	2.02	2.10	1.60	1.61	1.53

Table III: Effect of treating five day old P. boryanum cultures for five minutes with various concentrations of Triton X-100

	Triton X-100 Concentration						
	0	0.1%	0.25%	0.5%	1%	2%	10%
Microscopic Cell counts ( x 10 <sup>7</sup> cells per ml)	3.23	3.13	3.00	2.90	2.91	2.72	2.11

of nitric acid to use in the treatment to rupture the algal cells and release their ATP prior to measurement of ATP content in the population (Table IV). For subsequent experiments this 2.0N nitric acid concentration was used.

Figure 1 shows representative data in which the number of microscopic cell counts are shown for various dilutions of the five day old P. boryanum cultures. These counts were made without any prior treatment to disrupt the filaments. Figure 2 shows the average ATP values from 3 experiments obtained for various dilutions of the five day old algal population. Figure 3 shows representative data from a comparison of Spectronic 20 values for various dilutions of a five day old P. boryanum culture.

Figure 4 shows the relationship between Spectronic 20 readings and ATP values, Spec 20 readings and microscopic cell counts and ATP values and microscopic cell counts in various five day old cultures of P. boryanum.

The average ATP per cell value was obtained using all the data received from ATP values and microscopic cell counts. This value is  $6.94 \times 10^{-9}$   $\mu\text{g}$  ATP per P. boryanum cell. Blue-green algal cell volume as determined by the mean volume using the cylindrical and rectangular volume equations was  $4.70 \mu\text{m}^3$  per cell. The calculated ATP per volume value is  $1.48 \times 10^{-9}$   $\mu\text{g}$  ATP per  $\mu\text{m}^3$  for these Plectonema boryanum cells.

Table IV: The effect of various concentrations of Nitric Acid which is the rupturing agent of *P. boryanum* prior to measurement of ATP in the five day old population

Concentration of Nitric acid added for 5 minutes	ug ATP/ml Trial 1	ug ATP/ml Trial 2
1.5N	1.145	0.660
2.0N	1.291	0.802
2.5N	0.7937	0.434

Figure 1: Microscopic Cell Counts at Various Dilutions of Five day old *Plectonema boryanum* cultures

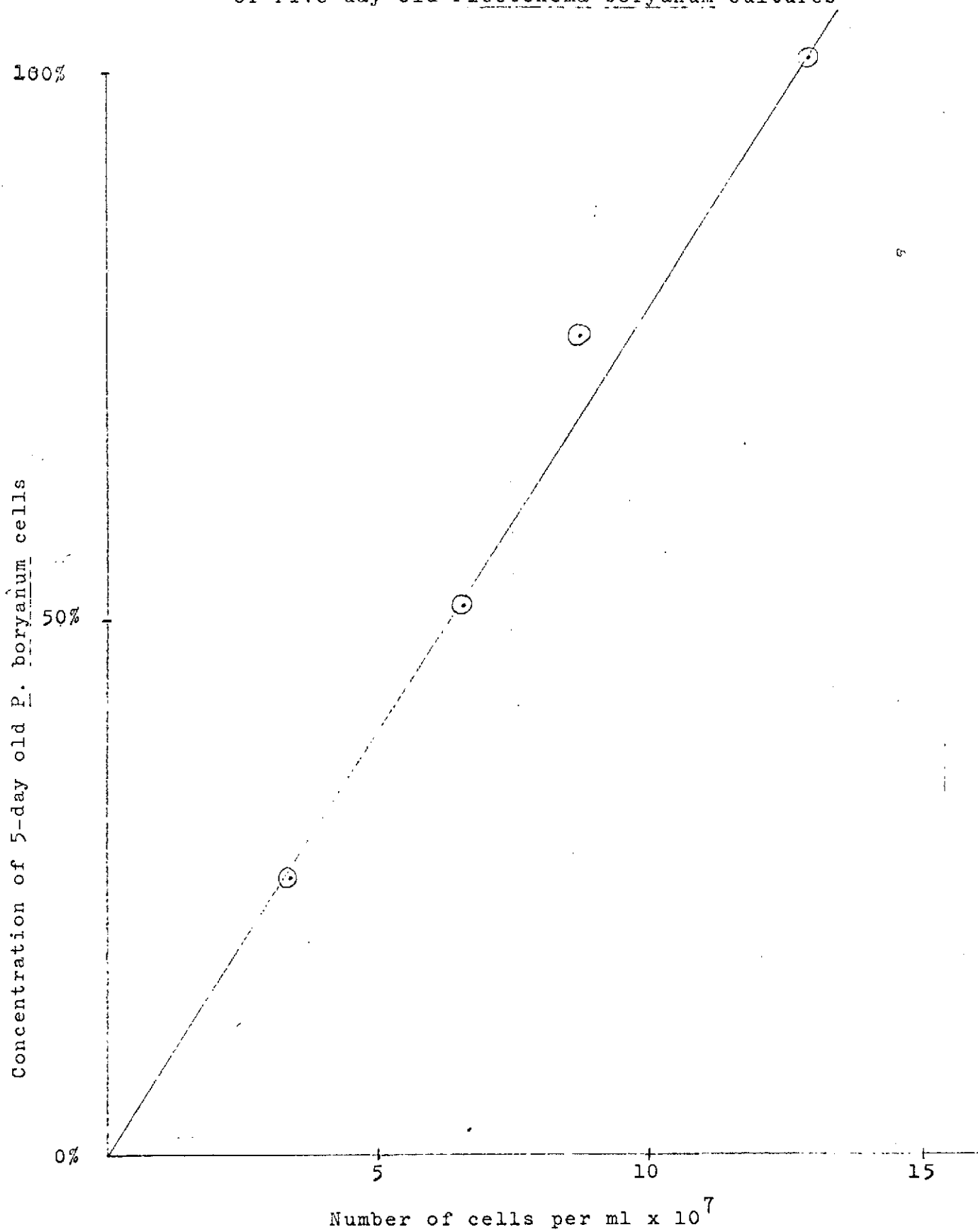


Figure 2: ATP Values at various dilutions of 5 day old *P. boryanum* cultures

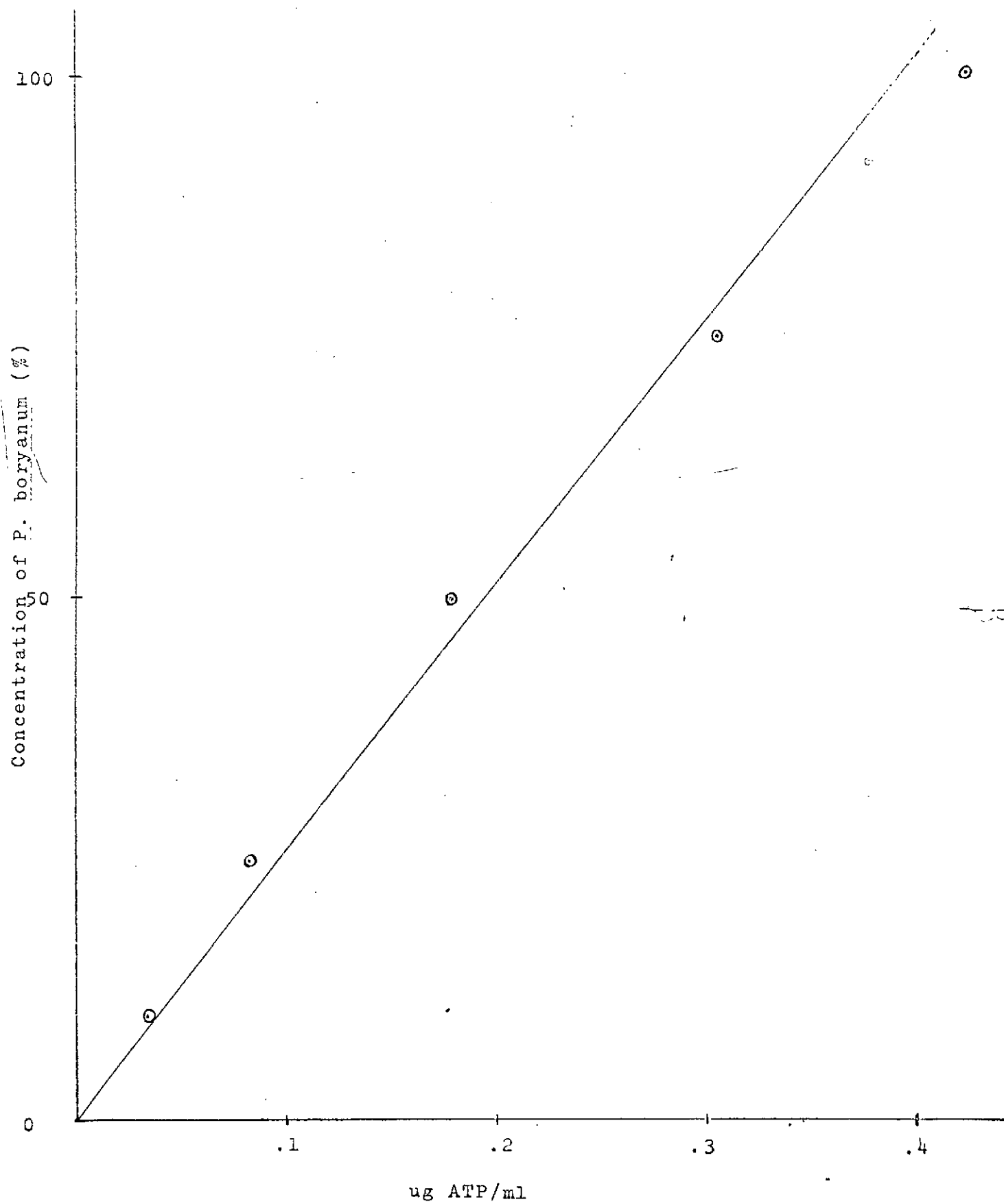


Figure 3: Spectronic 20 readings at various dilutions of five day old *P. boryanum* cultures

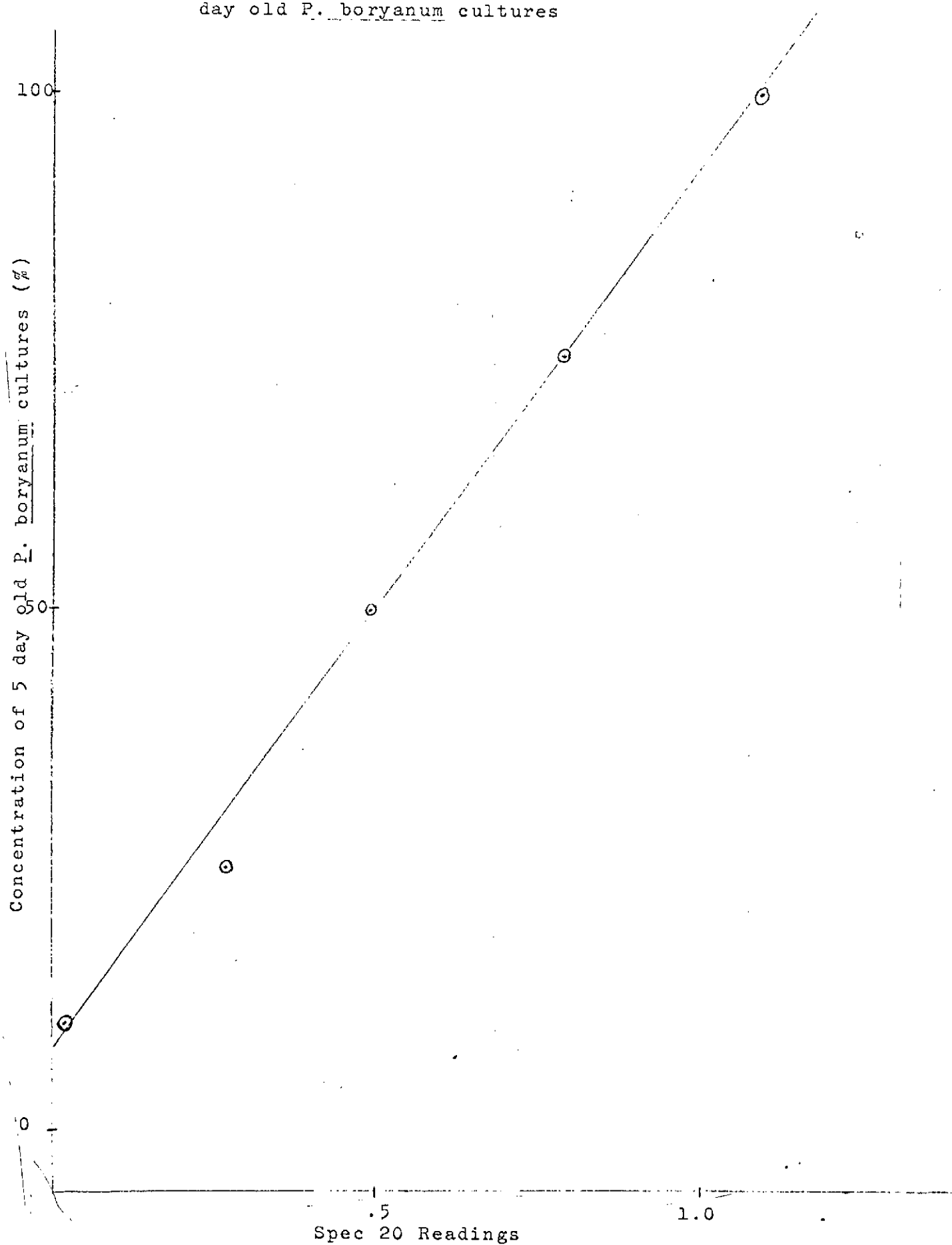
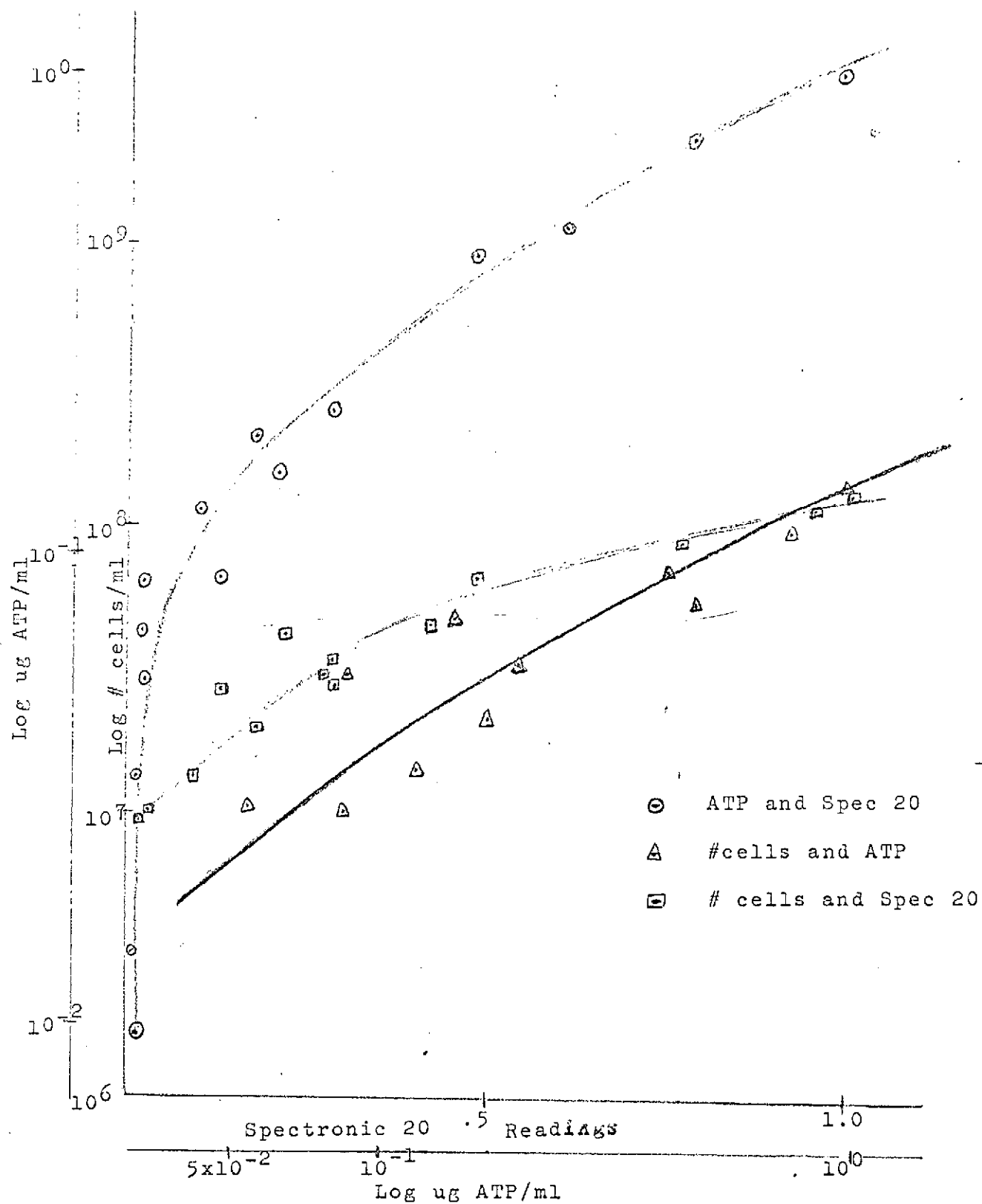


Figure 4: The relationship between Spectronic 20 readings, ATP values and microscopic counts in five day old *P. boryanum* cultures





## DISCUSSION AND CONCLUSIONS

Most of the previous work done with blue green algae has dealt with biochemical and morphological aspects of the cells. An accurate method for rapid titering of filamentous algae is needed. This study compares the more rapid luciferase assay for ATP with more conventional quantitation techniques.

Growth conditions were standarized so that the populations quantitated at various times would be as much alike as possible with respect to cell number and physiological state. We did find variation in cell number, Spec 20 values and ATP values in our five day old population on different days that the quantitation studies were made. The Climatarium was used as a growth chamber; room temperature changes at night may have influenced the growth rate of P. boryanum.

The various methods which were tried to disrupt the sheath join-  
ing the cells in the filament but not rupture the cell wall of the cells produced poor results. The various chemical treatments using pineapple juice, Triton X-100, nitric acid and sodium hydroxide either did not rupture the sheath or acted on both the sheath and cell wall. The chemical composition of the monosaccharides in the mucopolysaccharide sheath and wall appear to be similar in the species of blue-green algae which have been analyzed (Wolk, 1973). Sonication is probably one of the best ways to separate the cells provided individual cells are not ruptured. The setup devised for sonication for these experiments never broke the filaments apart as desired.

When the five day old population was diluted with growth medium, there was a linear relationship between each of the three methods and dilutions of the populations. In the case of ATP determinations, 2N nitric acid released more ATP in a given sample than other concentrations of nitric acid. All values obtained on five day old *P. boryanum* populations and the dilutions of these populations were used to see the relationship between Spec 20 values, log ug ATP/ml values and log of the number of cells/ml when cell number was determined by microscopic counting of unbroken filaments. At Spec 20 values above 0.1 the slopes of the graphs showing log ATP/ml vs. Spec 20 values and log of the number of cells/ml vs. log ATP/ml were similar; when the log of the number of cells/ml vs. Spec 20 values graph was compared with the two graphs discussed above, the slope at lower cell titers and Spec 20 values was similar, but then deviated at higher cell titers and Spec 20 values. In the two graphs in which ATP values were shown, they compared more favorably than when cell titers vs. Spec 20 was compared. The accuracy and rapidness of the luciferase assay for ATP makes it a technique which should be considered seriously as a titering technique.

ATP per cell values for a given species are dependent on the method of extraction of ATP used. With acid extractions of ATP, ATP per cell values increase as the volume of the cell used increases (bacteria  $10^{-10}$  -  $10^{-9}$ , blue-green algae  $7 \times 10^{-9}$ , yeast  $2.8 \times 10^{-8}$  ug ATP/ml) (Bush, 1973). If figured on an ATP/volume basis, the values for different types of cells are quite close. In this study such values for *P. boryanum* were calculated to be  $1.48 \times 10^{-9}$  ug ATP per  $\mu\text{m}^3$ . Bush (unpublished) found  $1.1 \times 10^{-9}$  ug ATP/ $\mu\text{m}^3$  for *Candida*.

albicans and  $1.8 \times 10^{-9}$  ug ATP/ $\mu\text{m}^3$  for 4 hour E. coli grown in trypticase soy broth.

This study was limited to P. boryanum of a given age and grown under specified conditions. Cells of varying ages and grown under different conditions should be examined to see how much variation in ATP/cell values exists. Holm-Hansen (1970) established that in an adequate nutritional environment, cellular levels of ATP are nearly identical in algal cells grown in the dark and in the light; changes in light intensity would not drastically affect ATP concentrations in the cell. He found temperature to cause fluctuations in ATP levels in cells. Once ATP/cell values are determined for a given situation the technique could be used with a minimum of effort for quantitation purposes.

#### LITERATURE CITED

- Bush, V.N. 1973. The effect of the presence of fungi, fungi-like bacteria and kidney cells on the examination of urine samples for pathogenic microbes by the luciferase assay for ATP. Del. State College Faculty J 4:12-20.
- Fay, P. and N.J. Lang. 1971. The heterocysts of blue-green algae. I. Ultrastructural integrity after isolation. Proc. Roy Soc. Ser.B 178:185-192.
- Holm-Hansen, O. 1970. ATP levels in algal cells as influenced by environmental conditions. Plant and Cell Physiol. 11:689 700.
- Kratz, W.A. and J. Myers. 1955. Nutrition and growth of several blue-green algae. Am. J. Bot. 42:282-287.
- McElroy, W.D. 1960. Emission and quantum yield of firefly bioluminescence. Arch. Biochem. and Biophys. 88: 136-141.
- Musbaum, V.F. 1970. Characterization of a possible lysogenic strain of the blue-green alga P. boryanum. Masters thesis, University of Delaware.
- Wolk, P.C. 1973. Physiology and cytological chemistry of blue-green algae. Bacteriol. Rev. 37(1):32-101.